Mature osteoclast–derived apoptotic bodies promote osteogenic differentiation via RANKL-mediated reverse signaling

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Supplementary Methods and Materials

Cell culture and Reagents

MC3T3-E1 cell line was obtained from American Type Culture Collection (ATCC). Recombinant Mouse RANKL and Recombinant Mouse M-CSF were purchased from R&D Systems (Minneapolis, MN). Antibodies against H2B (sc-515808), H3 (sc-56616), C3B (sc-28294), C1QC (sc-365301), CD9 (sc-13118), ACTB (sc-58673), ALP (sc-365765), COL1A1 (sc-293182), Osterix (sc-393060), RUNX2 (sc-101145), RANK (sc-59981), and GAPDH (sc-32233) were purchased from Santa Cruz Biotechnology (Santa Cruz). Antibody against p-PI3K (ab182651), PI3K(ab32089), p-Akt (ab81283), Akt (ab179463), p-S6K (ab59208), S6K (ab32529) was purchased from ABcam (Cambridge, UK). Cell Counting Kit-8 was obtained from Dojindo Molecular Technologies (Dojindo, Japan). TRAP stain kit was obtained from Sigma-Aldrich (NY, USA). Membrane dye DiI was obtained from Life Technologies. Alpha minimal essential Medium (α-MEM) and fetal bovine serum (FBS) was purchased from Gibco (life technologies, USA). Penicillin-streptomycin solution was obtained from Hyclone (Thermo Scientific, USA).

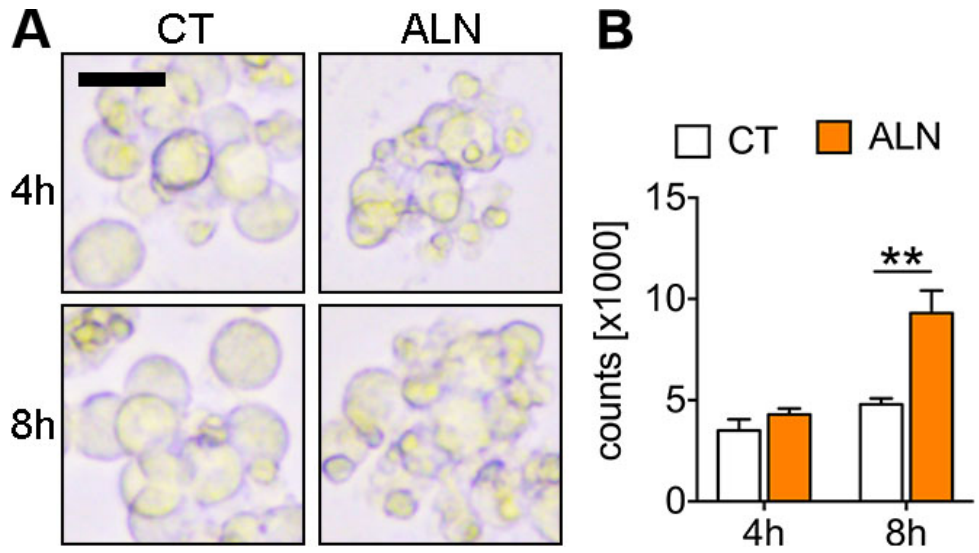
Cell viability assessment

Primary bone marrow monocytes/macrophages (BMMs) were seeded (2×10^3 per well) into 96-well plates and were cultured overnight. Cells were induced with M-CSF (50 ng/ml) and RANKL (100 ng/ml) for obtaining pOCs and mOCs. Cell proliferation and viability were evaluated by Cell Counting Kit-8 (CCK8, Dojindo, Japan) reagent at 0h, 24h, 48h, and 72 h

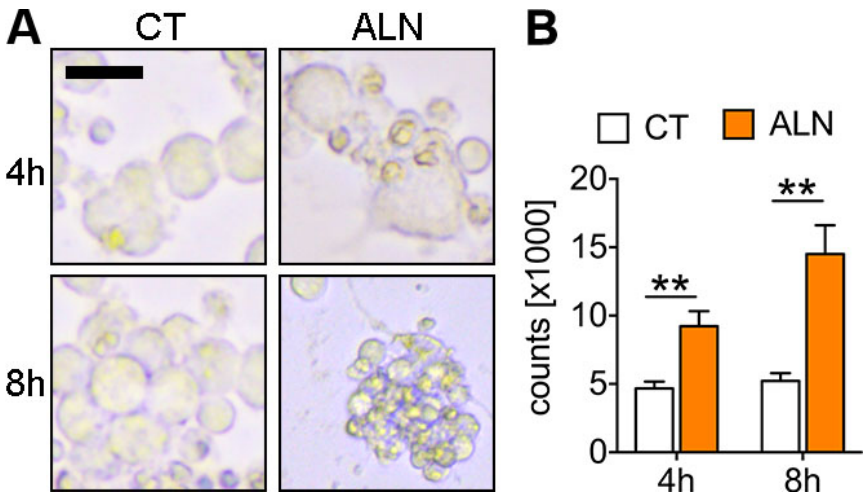
according to the manufacturers' instructions. The absorbency of cells was measured using a 96-well plate reader at 450 nm. Wells containing the CCK-8 reagent with no cells were used as the blank control.

Microscopy and confocal microscopy

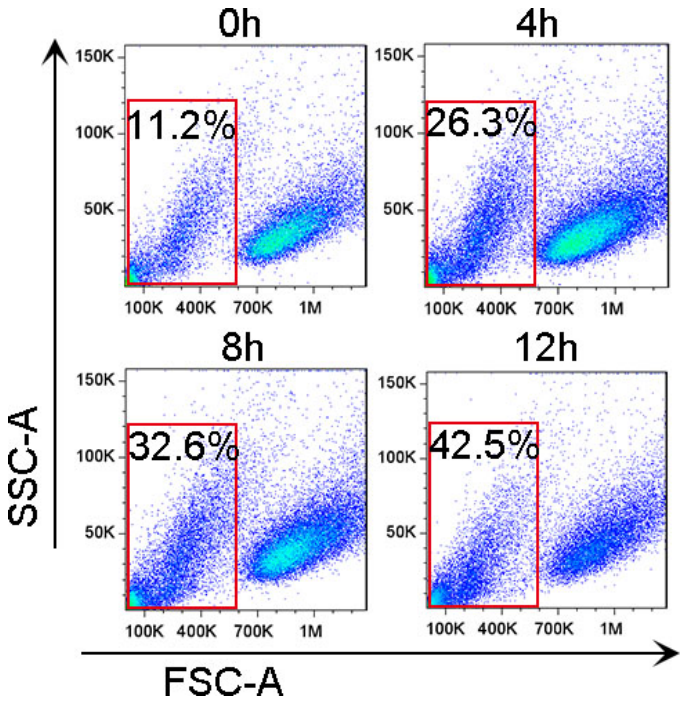
For light microscopy, cell morphology and state were observed by Olympus IX70 Inverted Microscope during cultured in 96-well plates or after trap staining. For confocal microscopy, cells were co-incubated with ABs in laser confocal dishes and analysis on Zeiss LSM800 using a 100x oil-immersion lens (excitation at 488, 568, 647 nm, detection at 650 nm, shown red and 488 nm, shown green). For analysis of engulfment, AnnexinV-FITC stained ABs were co-incubated with MC3T3-E1 (cultured for 24h), which were stained by cell tracker red.



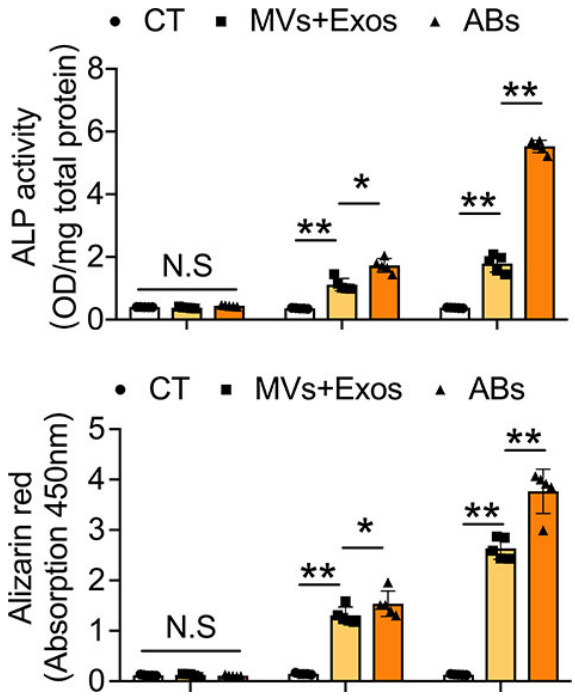
Supplementary Figure S1. a BMMs were induced with ALN (500 μM) and observed using light microscopy 4 and 8 hours after induction. Bar represents 20 μm. **b** Quantification of subcellular fragment counts. The data in the figures represent the averages ± SD. Significant differences are indicated as * ($p < 0.05$) or ** ($p < 0.01$) paired using Student's t test unless otherwise specified.



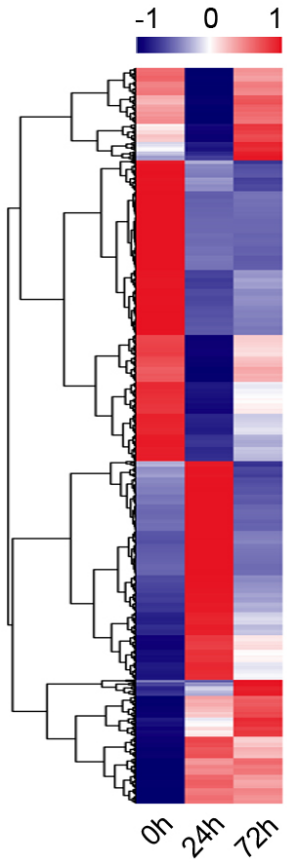
Supplementary Figure S2. a pOCs were induced with ALN (500 µM) and observed using light microscopy 4 and 8 hours after induction. Bar represents 20 µm. **b** Quantification of subcellular fragment counts. The data in the figures represent the averages ± SD. Significant differences are indicated as * ($p < 0.05$) or ** ($p < 0.01$) paired using Student's t test unless otherwise specified.



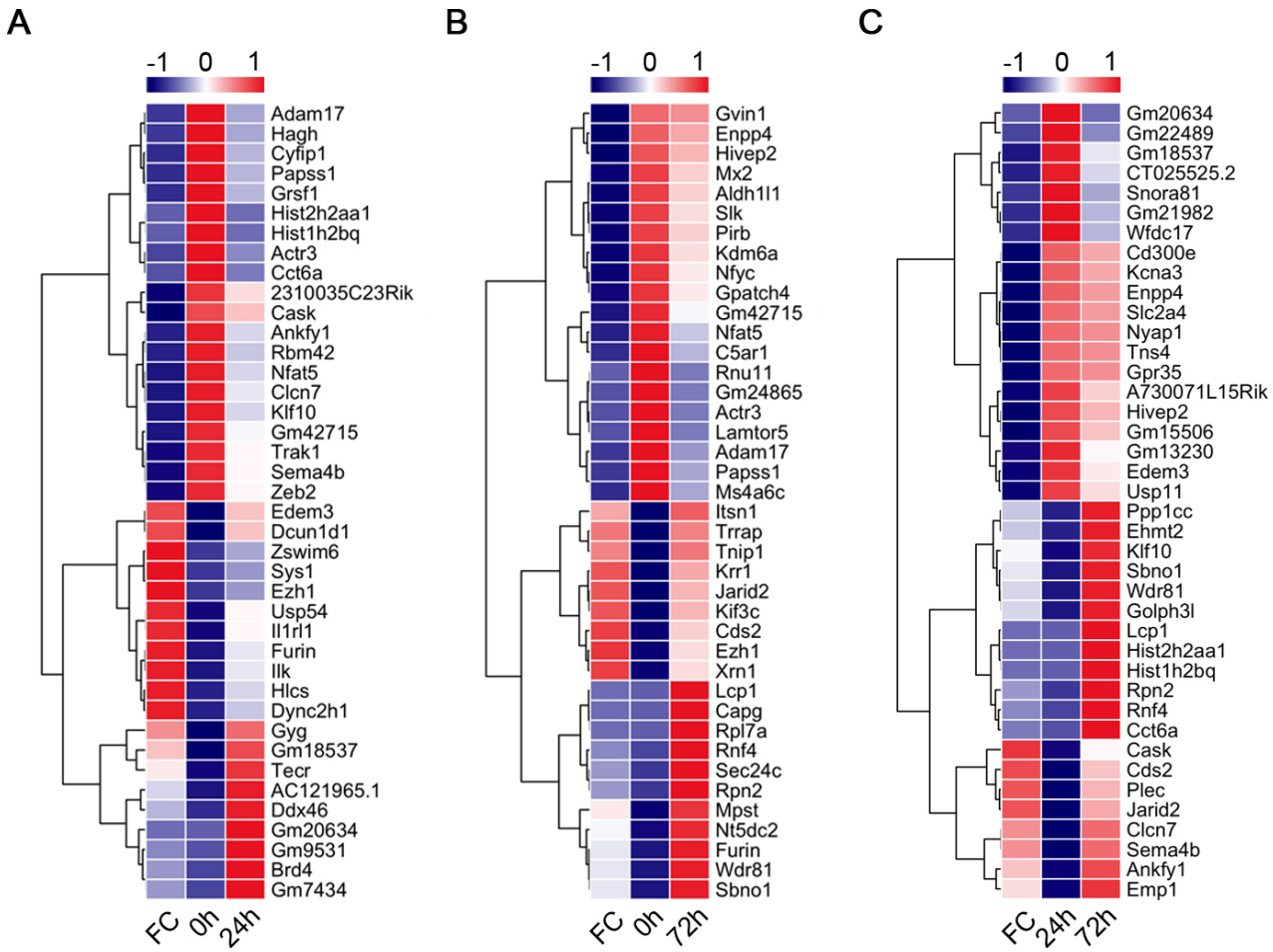
Supplementary Figure S3. Subcellular fragments containing ABs and MVs+Exos separated from apoptotic and viable cells by flow cytometry. Dot plots show FSC/SSC properties of apoptotic cells and subcellular fragments (circled population) after induction of apoptosis by ALN (500 μM). Subcellular fragments were quantified after the indicated incubation periods



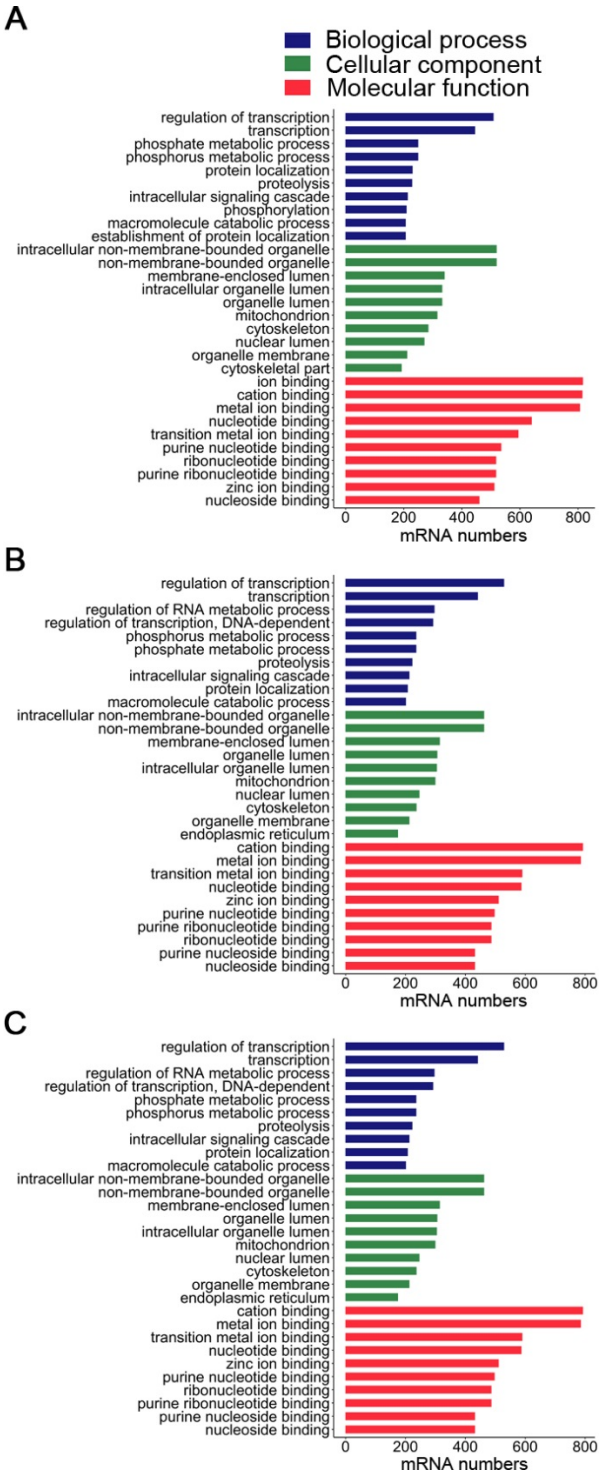
Supplementary Figure S4. Quantification of ALP activity and Alizarin red stain of MSCs treated with osteogenic factors in indicated groups. The data in the figures represent the averages ± SD. Significant differences are indicated as * ($p < 0.05$) or ** ($p < 0.01$) paired using Student's t test unless otherwise specified.



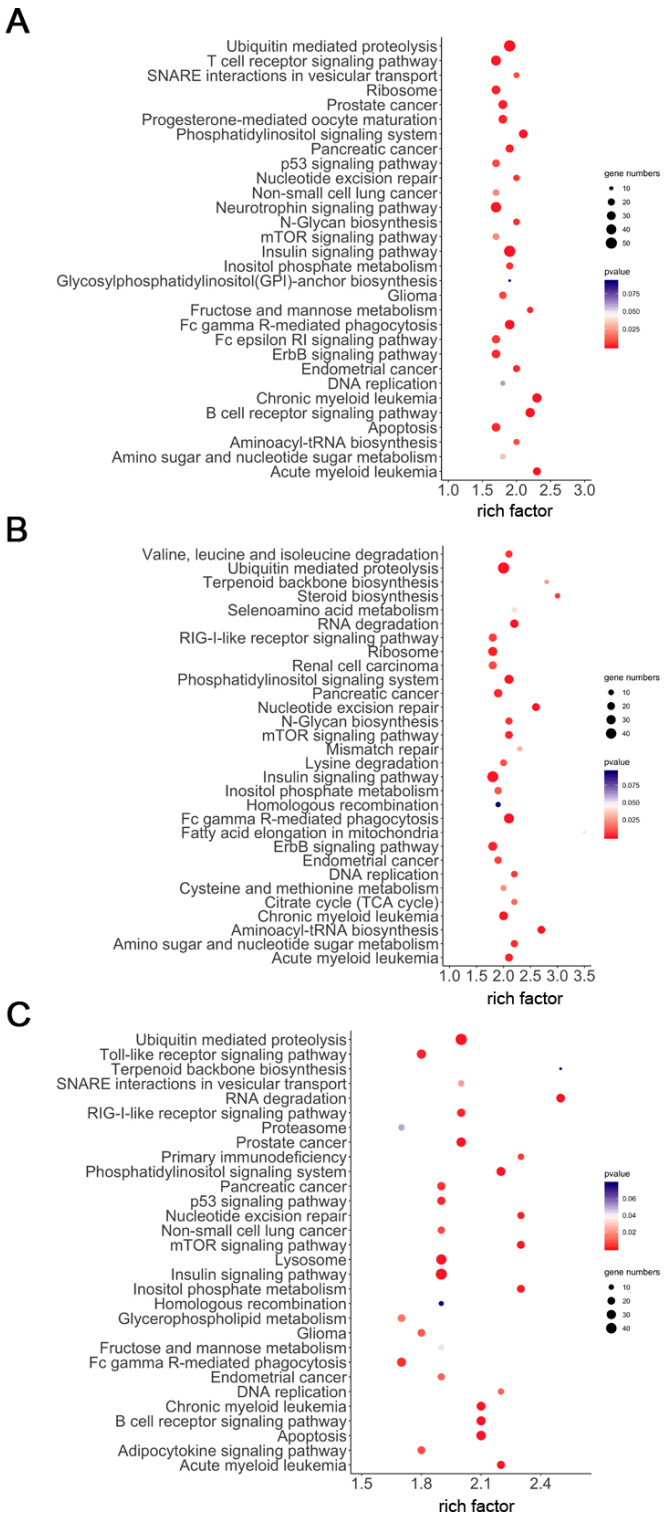
Supplementary Figure S5. Cluster heatmap showing all 14,196 differentially expressed mRNAs in BMM-ABs (0h), pOC-ABs (24h) and mOC-ABs (72h).



Supplementary Figure S6. Cluster heatmaps showing top 20 up and down regulated mRNAs in (A) BMM-ABs (0h) and pOC-ABs (24h), (B) BMM-ABs (0h) and mOC-ABs (72h), (C) pOC-ABs (24h) and mOC-ABs (72h).



Supplementary Figure S7. GO analysis of differentially expressed mRNAs in three groups. Top 10 BP, CC and MF terms for the differentially expressed mRNAs in (A) pOC-ABs and BMM-ABs, (B) mOC-ABs and BMM-ABs, (C) mOC-ABs and pOC-ABs.



Supplementary Figure S8. KEGG enrichment analysis of differentially expressed mRNAs in three groups. TOP 30 KEGG pathway terms of differentially expressed mRNAs in (A) pOC-ABs and BMM-ABs, (B) mOC-ABs and BMM-ABs, (C) mOC-ABs and pOC-ABs.

Supplementary Table S1. Primer sequences for qPCR

Genes	Forward	Reverse	Tm (°C)
RUNX2	5'-ATGCTTCATTCGCCTCACAAA-3'	5'-GCACTCACTGACTCGGTTGG-3'	61
ALPL	5'-AACCCAGACACAAGCATTCC-3'	5'-GAGACATTTTCCCGTTCACC-3'	60
COL1A1	5'-GCTCCTCTTAGGGGCCACT-3'	5'-ATTGGGGACCCTTAGGCCAT-3'	62
Sp7	5'-AAGTCTCAAGGTTATAGGGACGG-3'	5'-CCATGCTTGTCTGGGTATAGTGT-3'	62
GAPDH	5'-TGGATTTGGACGCATTGGTC-3'	5'-TTTGCACTGGTACGTGTTGAT-3'	60
β-actin	5'-TCCCTGTATGCCTCTG-3'	5'-ATGTCACGCACGATTT-3'	61